A Longitudinal Study to Identify IBS Phenotypes Using Fecal Microbiota and Hydrogen Breath Testing

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Title

A Longitudinal Study to Identify IBS Phenotypes Using Fecal Microbiota and Hydrogen Breath Testing

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SIGNATURE PAGE

The signature below constitutes the approval of this protocol and the attachments, and provides the necessary assurances that this study will be conducted according to all stipulations of the protocol, including all statements regarding confidentiality, and according to local legal and regulatory requirements and applicable US federal regulations.

Site Investigator: *

Signed: _____ Date: _____

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** The protocol should be signed by the clinical site investigator who is responsible for the day to day study implementation at his/her specific clinical site.*

LIST OF ABBREVIATIONS

IBS, Irritable Bowel Syndrome
SIBO, Small intestinal bacterial overgrowth
GHBT, Glucose hydrogen breath tests
FODMAP, Fermentable Oligosaccharides, Disaccharides, Monosaccharides, and Polyols
IBS-D, Diarrhea-predominant irritable bowel syndrome
VAS, Visual analog scale
NRS, Numerical Rating Scale
PHQ-9, Patient Health Questionnaire
VSI, Visceral Sensitivity Index
IBS-QOL, IBS-quality of life
TpH-1, Tryptophan hydroxylase-1
SNPs, Single nucleotide polymorphisms
OTU, operational taxonomic units
LEfSE, linear discriminant analysis effect size
AMOVA, analysis of molecular variance
eCRF, electronic Case Report Form
PHI, Protected Health Information
GCP, Good Clinical Practice
AE, Adverse Event

1 BACKGROUND/SCIENTIFIC RATIONALE

Irritable bowel syndrome (IBS) is a common but incompletely understood condition that leads to significant morbidity and substantial health care expenditures (1,2). IBS is likely a multifactorial disorder including disordered motility, immune activation, gut barrier dysfunction, visceral hypersensitivity, and altered brain-gut interactions (3–5).

The role of the gut microbiota is increasingly being recognized for its potential role *in the pathogenesis of IBS*. Humans are host to a diverse community of microbes collectively known as the human microbiota, of which the vast majority live in the gut (6). The gut microbiota normally interacts closely with the intestinal epithelial cells as well as the enteric immune and nervous system, which comprises the microbiota-gut-brain axis (7). There is a symbiotic relationship between the microbiota and host with the gut microbiome providing essential functions, including maintenance of the gut epithelial barrier and immune function (8). However, alterations in the structure and community function of the fecal microbiota may have a significant role in the pathogenesis of human disease, including IBS. Acute gastroenteritis leads to an increased risk for development of post-infectious IBS and is associated with altered gut microbiota, increased permeability and immune activation (9–11). Further evidence of the role of gut microbiota in IBS is shown by experiments where transfer of microbiota from patients with IBS to germ-free mice leads to rapid GI transit, impaired gut barrier function, and visceral hypersensitivity (12,13).

Several studies have reported an increase in the relative abundance of Firmicutes, a reduction in the relative abundance of Bacteroidetes, and decrease in microbial diversity (14–16). However, studies are often conflicting and a specific microbial signature has not been discovered to date in IBS (17–19). Incomplete phenotypic characterization, small sample sizes, the cross-sectional nature of study design, and probable heterogeneity of the patient populations also limited prior studies. In addition, previous studies have not taken differences in diet or stool consistency into consideration despite the fact that these factors heavily influence the composition of the gut microbiota (20,21).

Small intestinal bacterial overgrowth (SIBO) has also been postulated to be an *important pathophysiologic factor* in the pathogenesis of IBS (22–24). SIBO is defined as presence of bacteria in excess of 10^5 colony-forming units per milliliter on culture of small bowel aspirates [34]. Prevalence figures of SIBO in IBS range from 4-64% depending on the method of testing (26). SIBO is characterized by symptoms of diarrhea, abdominal pain, bloating, and gas. It has been proposed that SIBO may lead to immune activation, triggering low-grade inflammation, epithelial barrier dysfunction, and visceral hyperalgesia seen in IBS (27). Meanwhile, antibiotics improve symptoms in IBS patients with SIBO while those patients who achieved bacterial eradication showed the greatest symptomatic response (23). However, little is known about the specifics of the gut microbiota in SIBO and whether these conditions are separate or overlapping entities.

Small bowel aspirate and culture is an accepted means of quantifying upper intestinal bacteria, but this approach is time consuming, invasive, and has a high false-negative rate (28). As a consequence, hydrogen breath tests have largely supplanted intestinal cultures as measures of SIBO. There is some controversy regarding the substrate that is utilized in hydrogen breath

tests. Lactulose has been advocated as a preferred substrate as it is not absorbed in the GI tract. However, simultaneous testing using scintigraphy and lactulose hydrogen breath testing (LHBT) demonstrated that abnormal findings with LHBT represent rapid orocecal transit rather than SIBO (29). Glucose is another substrate that is commonly employed in hydrogen breath testing. Although glucose is primarily absorbed in the proximal small bowel and therefore may miss distal SIBO, this is still the most common and widely available non-invasive test to diagnose SIBO (30).

Despite our advances in understanding the pathophysiology of IBS, there are still **unmet needs** in terms of treatment options. Rifaximin is a non-absorbable antibiotic that has been shown to improve symptoms in the diarrhea-predominant subtype of IBS (IBS-D) (31,32). However, mechanisms by which rifaximin leads to improvement in IBS-D are still unknown. In vitro studies suggest that rifaximin may prevent changes in barrier dysfunction and reverse mucosal inflammation by modulating bacteria in the small intestine (33). Conversely, prior studies have not demonstrated significant effects of rifaximin on fecal microbial composition (34,35). This is likely because rifaximin is insoluble in the colon and has antimicrobial effects primarily in the small bowel (36). Furthermore, rifaximin leads to improvement in symptoms in *only 40% of IBS patients* with a therapeutic gain of 9% over placebo. Because of these observations, one could argue that rifaximin may be effective only in those IBS patients who have concomitant SIBO. Indeed, a recent study demonstrated that those IBS patients with SIBO had a significantly increased response to rifaximin compared with those IBS patients without SIBO (37).

Dietary modification has also been advocated as a potential treatment option in IBS-D. The vast majority of IBS patients believe certain foods trigger their IBS symptoms (38). Fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (FODMAP) are short-chain, poorly absorbable carbohydrates. Recently, a diet low in FODMAP has been shown to improve symptoms in up to 76% of IBS patients (39,40). However, two recent randomized controlled trials failed to show a low FODMAP diet was superior to traditional IBS dietary advice in controlling symptoms (41,42). Furthermore, improvement in symptoms was reported by *only 50% of patients on low FODMAP diets*. Moreover, the exact mechanism by which a low FODMAP diet improves symptoms in IBS is still unknown. It is postulated that the gut microbiota are critical in generation of symptoms in the presence of dietary FODMAPs and lead to production of gases, including hydrogen, CO2, and methane. This may generate symptoms in IBS particularly in the setting of visceral hypersensitivity (43).

Identifying those subjects who are *most likely to respond* to a low FODMAP diet is clearly needed but has been a vexing problem. Hydrogen breath testing has been advocated as a potential tool to identify subjects that malabsorb FODMAPs (e.g. lactose, fructose, sorbitol, and mannitol) and thereby might have better response to dietary FODMAP restriction. However, questions regarding the reproducibility of breath testing as well as standardization of the test in this setting are still unanswered (44). Furthermore, it was recently shown that a lactulose breath test could not discriminate between responders and non-responders to low FODMAP diets (45). Interestingly, a recent study in pediatric IBS subjects demonstrated that responders to a low FODMAP diet had *different fecal microbial composition* at baseline compared with non-responders (46). As the gut microbiota is postulated to be one of the main mediators in the pathogenesis of symptoms in IBS and FODMAP intake, this suggests that alterations in the fecal microbiota may predict response to dietary restriction of FODMAP. Genetic variability in

tryptophan hydroxylase-1 (TpH-1) and sucrase-isomaltase single nucleotide polymorphisms (SNPs) have been demonstrated in IBS patients (47,48). Unpublished data suggest that differences in TpH-1 SNPs may also potentially identify responders vs. non-responders to treatments, such as low FODMAP diet, in IBS. Finally, a recent study suggested that treatment with low FODMAP diet may lead to improvement in cytokine profiles (49).

Taken collectively, our current therapeutic options are quite limited in IBS-D and improve symptoms in only $\leq$ 50% of subjects in the best-case scenarios, which we believe is a consequence of multiple pathogenic factors. Given that IBS is a heterogeneous condition, there is a **pressing need to stratify IBS patients into distinct subtypes** to optimize responses to diverse therapies including antibiotics versus dietary measures. In the investigations included in this proposal, we will test the hypotheses that: (i) distinct IBS-D phenotypes with characteristic clinical features can be generated by identifying the presence or absence of SIBO and by defining fecal microbial populations, and (ii) longitudinal analyses using microbiota-derived metrics as well as SIBO status may provide important details into how these treatments improve symptoms in IBS and may also provide clues on which patients may respond to different therapies.

2 OBJECTIVES

Aim 1: Randomize IBS-D subjects to receive either treatment with a nonabsorbable antibiotic rifaximin vs. low FODMAP dietary intervention.

a) 200 IBS-D patients will be carefully phenotyped using validated questionnaires (IBS symptoms, stool form, psychological function, quality of life) and diet analysis.

b) IBS-D subjects will undergo baseline testing including glucose hydrogen breath tests (GHBT) to define the presence of SIBO. Fecal microbiota will also be characterized by α- and β-diversity and dominant phyla on 16S RNA analyses.

c) IBS-D subjects will be randomized to receive either rifaximin 550 mg three times daily x 14 days or low FODMAP diet x 4 weeks under direction of study bionutritionist.

d) The primary outcome will be changes in mean daily pain or bloating by visual analog scale (VAS) after intervention compared with baseline. Responders to intervention will be defined by $\geq$ 30% reductions in mean daily pain or bloating by VAS compared with baseline. Secondary outcomes will be defined by reduction in IBS Symptom Severity Scale by $\geq$ 50 compared with baseline.

e) Other secondary outcomes, including GI symptoms, stool form, psychological function, quality of life, and diet intake, will be compared to determine if subjects present differently.

Aim 2: Determine using longitudinal analyses how SIBO status and fecal microbiota features associate with response to treatment with rifaximin or a low FODMAP dietary intervention.

a) Fecal microbial analyses will be repeated at 2, 4 and 5 weeks while GHBT will be repeated at the end of the intervention.

b) The primary aim will be change in fecal microbial diversity after intervention compared with baseline. We hypothesize that low FODMAP diet will result in a 10-15% increase in diversity in fecal microbiota compared with baseline.

c) Secondary outcomes including response to treatment with rifaximin or low FODMAP diet will be related to the (i) presence of SIBO on initial testing, (ii) persistence of SIBO after intervention, (iii) abnormal α- and β-diversity on initial testing, (iv) changes in α- and β-diversity as well as in dominant microbial phyla at 2, 4 and 5 weeks, and (v) changes in serum cytokine profiles, including TNF-α, IL-1β, IL-6, IL-8, and IL-10.

d) Other outcomes that will be assessed include differences in tryptophan hydroxylase-1 (TpH-1) and sucrase-isomaltase single nucleotide polymorphisms (SNPs) in different cohorts of IBS-D patients.

Aim 3: Develop a predictive model to identify subsets of IBS-D patients responsive to treatment.

a) We will develop and validate a predictive model of response to low FODMAP diet using data from the subjects randomized to a low FODMAP diet (Aim 1) combined with existing data from a separate cohort of IBS-D subjects previously treated with a low FODMAP diet (HUM 00053274).

3 EXPECTED RISKS/BENEFITS

The data accumulated in this study will advance our understanding of the pathophysiology of IBS, including the role of the gut microbiota and SIBO in the pathogenesis of IBS, and may provide important information on which IBS subjects will most benefit from therapies including dietary modification and rifaximin.

There are potential direct benefits for all IBS subjects in this study. On study entry, glucose hydrogen breath tests (GHBT) as well as analysis of fecal microbiota will be performed that may provide new insight into causes of symptoms in patients with IBS. Furthermore, subjects with IBS will be treated with well accepted treatments for IBS, including a nonabsorbable antibiotic rifaximin or diets low in FODMAP. IBS-D subjects treated with low FODMAP diet will be provided education on a low FODMAP diet from a trained bionutritionist. IBS-D subjects will track their symptoms by completing questionnaires online including the Bristol Stool Form Scale as well as an 11-point NRS on abdominal pain and bloating daily. IBS-D subjects will be asked to complete these questionnaires after their evening meal. They will also be instructed to track their diet for 24 hours prior to each weekly or biweekly visit using a food questionnaire. Finally, all IBS-D subjects will be seen or receive a phone call by the PI and/or study team every week or every two weeks during the study. At each visit, survey information will be obtained using validated questionnaires including IBS-Symptom Severity Scale, Gastrointestinal Symptom Rating Scale-IBS, Patient Health Questionnaire (PHQ-9), Visceral Sensitivity Index (VSI), and IBS-quality of life (IBS-QOL). The IBS-Symptom Severity Scale is a 10-item questionnaire that tracks severity of IBS symptoms and is a

valid way to monitor changes in IBS symptoms over time. This will take approximately 5 minutes to complete. The Gastrointestinal Symptom Rating Scale-IBS is a 15-item questionnaires that tracks both upper and lower gastrointestinal symptoms. This will take approximately 8 minutes to complete. The PHQ-9 is a 9-item questionnaire that is a reliable and valid measure of depression severity. This will take approximately 5 minutes to complete. The VSI is a 15-item questionnaire which is a reliable, valid measure of GI-specific anxiety. This will take approximately 8 minutes to complete. The IBS-QOL is a 34-item questionnaire that is a reliable, valid quality of life measure specific for IBS. This will take approximately 15 minutes to complete. This frequent and comprehensive assessment provided as part of participating in this study may allow for closer monitoring of disease course.

There are potential risks for subjects, including:

1) Risk to privacy (rare): There is a potential risk to the IBS-D subject's privacy (<1% risk). While every effort will be made to maintain privacy, it is possible that others may learn about information acquired from the medical records.

   For Aim 3, there are additional risks to privacy associated with secondary review of data from a separate database (HUM 00053274). However, we will follow standard procedures to minimize these risks. This includes use of data abstraction forms that will not contain any PHI. All subjects will be identified by a study ID number. The list linking the subject to his/her study ID number will be retained by the investigators in a secure location separate from the source documents. The list linking PHI to study ID numbers will be accessed only for verification of data abstraction if any inconsistency or incompleteness of study data requires review of a participant's medical record. Once the data has been reviewed, analyzed, and verified, and the investigators are satisfied that no further review of the subject's medical record is needed, the list of links to the identifiers for that subject population will be destroyed. This will ensure that no links to PHI persist beyond the duration of the study. No PHI will be sent outside of the institution. All presentations/publications will deal with data in aggregate form. Source documents created for study purposes will identify patients by study ID number only and will not contain PHI.

2) Glucose hydrogen breath testing (likely): IBS-D subjects will be asked to drink 75 g of glucose dissolved in 250 ml of sterile water. IBS-D subjects may experience (10-25%) bloating, abdominal cramping, and/or flatulence after consuming this drink. However, symptoms typically resolve quickly and may represent underlying physiologic abnormalities causing their symptoms. Subjects will be allowed to take medications, such as simethicone or Tylenol, if they experience significant bloating or discomfort. IBS-D subjects may experience dizziness, faint spells, and/or nausea after the glucose hydrogen breath test. Subjects should bring a snack with them on

days that the hydrogen breath test should be done to help with dizziness and lightheadedness.

3) Risks of Blood Draw: If subjects decide to participate in optional sub-study, 10mL of blood will be drawn from a vein at Visits 1 and 5. There could be pain, bruising, or lightheadedness. Rarely there could be an infection or fainting. The person drawing blood will be trained to do that procedure and use techniques to minimize these risks.

4) Risks of treatment (infrequent): There are theoretical concerns about treatment with rifaximin and low FODMAP diet. Rifaximin is an antibiotic and theoretical concerns exist regarding potential for antibiotic resistance. However, rifaximin is a minimally absorbable antibiotic with low risk of clinically relevant bacterial resistance (50,51). Randomized controlled trials with rifaximin in IBS have not demonstrated significant differences in adverse events between rifaximin and placebo (31,32,52). General antibiotics side effects may occur, such as vomiting, nausea, diarrhea, etc. There are also theoretical concerns that prolonged therapy with a low FODMAP diet may have detrimental effects on gut microbial composition (53). However, multiple studies examining low FODMAP diet in IBS have demonstrated the safety and tolerability of this dietary approach (40–42,45). The effects of rifaximin in pregnancy and women who are breastfeeding are unknown and therefore pregnant women or women who are breastfeeding will be excluded from the study. Finally, women of child bearing age will be warned that oral contraception may not be as effective when taken with rifaximin.

5) General risks (infrequent): This is an interventional study. IBS-D subjects' condition may worsen or improve while undergoing intervention with either rifaximin or a low FODMAP diet. Participation in this study will not prevent normal clinical care of IBS or its consequences. In addition, some prescribed medications may need to be stopped temporarily during the study. This includes medications that may influence pain sensation, including narcotic pain medications. IBS-D subjects will also be asked to discontinue antibiotics for at least 3 months and probiotics for at least 1 month prior to enrollment. IBS-D subjects will be asked to avoid Pepto-Bismol for at least 2 weeks prior to GHBT. IBS-D subjects will also be instructed to eat a low carbohydrate diet for at least 2 days prior to the GHBT to avoid inaccurate test results. If IBS-D subjects are taking any of these medications, their symptoms may return or worsen when they are stopped. However, the stopping of medications is temporary and is not felt to cause any undue hardship. Finally, there is a risk that some of the questions in the surveys may produce emotional distress. If the IBS-D subject desires, he/she can be referred to

a mental health specialist within the UMHS or another provider outside the UMHS system.

6) Risks for sharing samples (rare): If subjects decide to participate in an optional sub-study, saliva samples will be shared with Karolinska Institute and IKMB Kiel to evaluate differences in sucrase-isomaltase SNPs. There are potential risks to the IBS-D subject's privacy. However, all samples will be de-identified and only investigators at UMHS will have access to the linkage code.

4 ELIGIBILITY CRITERIA

IBS Patients:

200 consecutive adult subjects ≥ 18 years of age at screening who meet the following inclusion and exclusion criteria will be eligible for enrollment:

Inclusion criteria:

- Rome IV criteria for IBS-D
- Subjects will be asked to avoid Pepto-Bismol for 2 weeks prior to GHBT.
- Subjects will be instructed to avoid eating or drinking for 8 hours and avoid smoking for 6 hours prior to the test
- Have colonoscopy or sigmoidoscopy with mucosal biopsy within the past 2 years prior to enrollment
- Have an average score of 4 or higher over a 7-day pre-screen taking online symptom surveys assessing abdominal pain and bloating

Exclusion criteria:

- Underlying celiac disease
- Inflammatory bowel disease
- Other organic disease that could explain their symptoms.
- History of GI tract surgery, except for appendectomy
- Narcotics, antibiotics taken within 3 months or probiotics taken within one month prior to enrollment will not be permitted.
- Previously received formal dietary education, including a low FODMAP diet, for treatment of IBS.
- Previously received antibiotics, such as rifaximin, for treatment of IBS or SIBO.
- Women who are pregnant
- Women who are breastfeeding

Additional IBS subjects and Healthy Controls:

50 consecutive adult subjects ≥ 18 years (25 IBS-D and 25 healthy controls) who are undergoing colonoscopy for clinical purposes (e.g. colon cancer screening)

For IBS subjects:

Inclusion criteria:

- Rome IV criteria for IBS-D

Exclusion criteria:

- Underlying celiac disease
- Inflammatory bowel disease
- Other organic disease that could explain their symptoms.
- History of GI tract surgery, except for appendectomy
- Narcotics, antibiotics taken within 3 months or probiotics taken within one month prior to enrollment will not be permitted.
- Women who are pregnant
- Women who are breastfeeding

For healthy controls:

Exclusion Criteria:

- History of chronic GI disorder, such as irritable bowel syndrome, inflammatory bowel disease, and small intestinal bacterial overgrowth
- Narcotics, antibiotics taken within 3 months or probiotics taken within one month prior to enrollment will not be permitted
- Women who are pregnant
- Women who are breastfeeding

5 SUBJECT ENROLLMENT

Most patients with irritable bowel syndrome are seen in gastroenterology or general medicine clinics. IBS-D patients will be recruited from gastroenterology and general medicine clinics at Taubman Center and in the satellite clinics within the University of Michigan Health System. We will also be sending out letters to patients identified from DataDirect as eligible for the study. Eligible IBS-D subjects will be contacted and given material explaining the nature of the study and introduction to a low FODMAP diet. They will be given time to read the materials. If they choose to participate, they will undergo a one week pre-screening an 11-point numerical rating scale (NRS) for abdominal pain and bloating for 7 days. If the subjects qualify through the pre-screen, they will sign the consent document in the presence of the PI and/or Designee. IBS-D subjects will be enrolled on a first come, first served basis. This approach will ensure that IBS-D subjects are representative of the population of patients with IBS in Southeast Michigan.

Emails to providers in the General Medicine and Gastroenterology clinics in the University of Michigan System, public advertisements, telephone calls, and web postings on UMClinicalStudies.org will also be utilized to recruit IBS-D subjects.

IBS subjects who do not meet inclusion/exclusion criteria will not be enrolled in the study. All data collected from IBS-D subjects who are screen failures will be saved for data analysis.

IBS subjects and healthy controls who are undergoing colonoscopy will be approached in the Medical Procedures Unit to determine their interest in participating in the optional sub-study and to determine their eligibility.

6 STUDY DESIGN AND PROCEDURES

Specific Aim 1: Randomize IBS-D subjects to receive either a nonabsorbable antibiotic rifaximin vs. low FODMAP dietary intervention.

1A. Research Approach:

200 IBS-D subjects will be carefully phenotyped using validated questionnaires (IBS symptoms, stool form, psychological function, quality of life) and diet analysis. IBS-D subjects will also undergo baseline testing including GHBT as well as fecal microbial analysis. IBS-D subjects will then be randomized to receive either rifaximin or low FODMAP diet intervention at the initial screening visit. Efficacy of therapies will be monitored with validated questionnaires to track symptomatic improvement. The primary outcome will be changes in mean daily pain or bloating after intervention compared with baseline. Responders to intervention will be defined as ≥ 30% reduction in mean daily pain or bloating by visual analog scale (VAS) compared with baseline. Secondary outcomes will monitor changes in IBS-Symptom Severity Score as well as differences in GI symptoms, stool form, psychological function, quality of life, and dietary intake before and after intervention.

1B. Experimental Methods:

Subject Recruitment: 200 adult subjects ≥ 18 years of age who meet Rome IV criteria for IBS-D will be enrolled in the study. Women of childbearing age will undergo a urine pregnancy test to exclude the possibility of pregnancy. Women who are breastfeeding will also be excluded. IBS-D subjects will be excluded if they have underlying celiac disease, inflammatory bowel disease, or other organic disease that could explain their symptoms. All IBS-D subjects will have prior sigmoidoscopy or colonoscopy with mucosal biopsies within the past 2 years to rule out microscopic colitis. IBS-D subjects with a history of GI tract surgery, except for appendectomy, will also be excluded from the study. Antibiotics taken within 3 months prior to enrollment will not be permitted (54). IBS-D subjects on probiotics must discontinue their use at least 1 month prior to fecal collection. IBS medications, including anti-depressants, will be allowed if the dose has been stable for at least 1 month before inclusion. Medications will be carefully tracked to follow any potential confounding issues. IBS-D subjects who have previously received formal

dietary education for IBS, including a low FODMAP diet, or previously received antibiotics, including rifaximin, for treatment of IBS-D or SIBO will be excluded from the study.

GI Symptom Assessment: All IBS-D subjects will complete validated questionnaires to quantify severity of IBS symptoms, including IBS Symptom Severity Scale (IBS-SSS), Gastrointestinal Symptom Rating Scale-IBS (GSRS-IBS), an 11-point numerical rating scale (NRS) for abdominal pain and bloating, and Bristol Stool Form Scale (BSFS) at baseline (55–58). The NRS for abdominal pain and bloating and the BSFS will be completed online through the REDCap Survey Tool.

Psychological and Quality of Life Assessment: IBS-D subjects will also complete validated questionnaires to assess for presence of and to measure severity of psychological disorders, GI specific anxiety, and impairments in quality of life. All IBS-D subjects will complete the Patient Health Questionnaire (PHQ-9), Visceral Sensitivity Index (VSI), and IBS-quality of life (IBS-QOL) (59–61). The PHQ-9 will be reviewed by the study team within 24 hours of completing the questionnaire. If a subject answers affirmative to the question "Thoughts that you would be better off dead or of hurting yourself in some way," they will be provided with the appropriate referral to mental health counseling and/or the subject's PCP will be alerted.

Dietary Assessment: All IBS-D subjects will undergo initial consultation with the study team and will be instructed on how to monitor and record their dietary intake. IBS-D subjects will then record daily their dietary intake for 3 days prior to fecal collection.

Fecal Collection and Preparation: Three fresh stool samples will be collected from all IBS-D subjects at baseline. Each sample will be immediately placed into an OMNIgene•GUT collection tube (DNA Genotek, Inc., Ottawa, ON, Canada) which allows for self-collection and stabilization of fecal DNA samples for up to 60 days at ambient temperature (62). Samples will then be mailed back or picked up by the study team and stored at -80^{o}C in Vince Young's Lab for long-term storage. If patients consent to the optional portion of the study, they will provide a stool sample in 95% ethanol at Visits 1, 3, 4, and 5. We will also collect a one-time stool sample and symptom questionnaires from healthy controls. Healthy controls will not have a history of gastrointestinal conditions and do not report any abnormal bowel movements or abdominal pain.

Tryptophan hydroxylase-1 (TpH-1) and sucrase-isomaltase single nucleotide polymorphisms (SNPs): Buccal swabs will be collected from all IBS-D subjects at baseline. Each sample will be immediately placed into an Oragene•DISCOVER (DNA Genotek, Inc., Ottawa, ON, Canada) which allows for stabilization of salivary DNA samples for up to 5 years at ambient temperature. Samples will then be stored at -80^{o}C for long-term storage. De-identified DNA samples will be shared with Karolinska Institute and IKMB Kiel to analyze differences in sucrase-isomaltase SNPs. This portion of the study is optional and subjects have a choice to allow their saliva samples to be stored for this sub-study.

Serum cytokine profiles: Serum cytokines, including TNF-α, IL-1β, IL-6, IL-8, and IL-10 will be measured at baseline and at week 5. Whole blood will be collected in a 10 ml SST tube. SST tubes will be allowed to clot at room temperature for 30 minutes. The serum will then be separated from the red blood cells by spinning for 15 min at 1300G at 4^{o}C. The serum will then be aliquoted from each blood collection tube into (3) 2-ml transfer tubes and stored at -80^{o}C for

future analyses. This portion of the study is optional and subjects have a choice to allow their blood samples to be stored for this sub-study

Glucose Hydrogen Breath Test (GHBT): SIBO will be diagnosed using the glucose H_2/CH_4 breath test as previously described (63). Briefly, H_2/CH_4 breath concentrations will be measured by gas chromatography (Quintron Instrument Company, Milwaukee, USA) in parts per million (ppm) after administration of an oral loading dose of glucose (75 g in 250 ml of sterile water). Breath samples will be collected every 15 minutes for a total of 120 minutes. The test will be considered positive for SIBO when H_2 and/or CH_4 increase by $\geq$ 12 ppm above baseline. COVID-19 modifications: Patients will be given take home hydrogen test kits either in person or through the mail to conduct the test at home. Instructions will be given prior to test with the take home test kit. Patients could bring the test result back either in person or through mail and the lab will analyze the results.

Trial Protocol: In order to minimize unequal allocation between arms, IBS-D subjects will be randomized using block randomization with random permuted sizes of 2 or 4 in a 1:1 ratio into two arms: one group will undergo treatment with rifaximin 550 mg three times daily for 14 days. The second group will be placed on a low FODMAP diet (≤ 9 grams per day as previously described) for 4 weeks (40,64,65). IBS-D subjects undergoing dietary intervention will undergo consultation with a bionutritionist knowledgeable in low FODMAP diets. A diet plan will be implemented and individualized to subjects based on information from their dietary history. Written information regarding allowable vs. non-allowable food items will be provided to IBS-D subjects and bionutritionists will be available during the study for further education. IBS-D subjects randomized to the low FODMAP diet group will meet with the GI nutritionist at the completion of the 2nd week of dietary intervention to answer any potential questions regarding the diet. IBS-D subjects will complete a 24-hour dietary recall each week or every two weeks during the 4-week dietary intervention to ensure compliance.

Symptom Assessment: IBS-D subjects will record stool frequency, BSFS, and symptom scores for abdominal pain and bloating daily on a diary card. IBS-D subjects will complete questionnaires on a weekly basis during study visits to determine changes in symptoms. Responders to intervention arm (both rifaximin and low FODMAP diet) will be defined as ≥ 30% reductions in mean daily pain or bloating compared with baseline. Secondary outcomes, including reduction in IBS Symptom Severity Scale by $\geq$ 50 compared with baseline at the end of the interventional arm, will also be monitored. These are both considered to reflect clinically meaningful improvements in IBS (55,57). IBS-D subjects will be seen on a weekly or biweekly basis and diary cards will be collected. Adherence to medications or dietary intervention will also be assessed at that time.

Specific Aim 2: Determine using longitudinal analyses how SIBO status and fecal microbiota-derived metrics associate with response to treatment with rifaximin or a low FODMAP dietary intervention.

2A. Research Approach:

We will prospectively follow the results of GHBT and fecal microbiota before and after treatment with rifaximin and low FODMAP dietary restriction. We will collect fecal samples throughout the study period in IBS-D patients. All IBS-D subjects will also undergo GHBT before and after

intervention. The primary aim will be change in fecal microbial diversity after intervention with rifaximin or low FODMAP diet compared with baseline. Secondary outcomes will evaluate whether response to treatment with rifaximin or low FODMAP diet is associated with: (i) presence of SIBO on initial testing, (ii) persistence of SIBO after intervention, (iii) changes in α- and β- diversity as well as in dominant phyla. We will also utilize human intestinal enteroids to model how host-microbial interactions may influence response to treatment with rifaximin or low FODMAP diet in IBS.

2B. Experimental Methods:

Fecal Collection and Preparation: Stool samples will be collected from all IBS-D subjects at weeks 2, 4 and 5 as described in Aim 1. Stool samples will be stored for up to 5 years at the conclusion of the study for future analyses, including evaluating differences in metabolomics, such as histamine, p-hydroxybenzoic acid, azelaic acid, and H_2 (45). This portion of the study is optional, and subjects have a choice to allow their stool samples to be stored for this sub-study. This may provide mechanistic descriptions of how changes in gut microbiota lead to changes seen in IBS. If patients consent to this optional portion of the study, they will provide a stool sample in 95% ethanol at weeks 0, 2, 4, and 5. Subjects will be compensated a nominal amount of $20 for stool samples returned at week 0 and $10 each for weeks 2, 4 and 5 to express our gratitude for their participation in the study. We will also provide mailers to mail back samples to minimize study burden on participants.

GHBT: Glucose H_2/CH_4 test will be repeated at week 5 as described in Aim 1.

Serum cytokine profiles: Serum cytokines will be repeated at week 5 as described in Aim 1. Handling and processing of the blood will be described in Appendix 1.

Mucosal Samples: If subjects consent to participate in this optional sub-study, mucosal biopsies will be taken from eligible subjects who are scheduled to undergo a colonoscopy for clinical purposes (e.g. to rule out alternative causes of symptoms or screening for colon cancer). In addition to the IBS subjects recruited to participate in the clinical trial, we will recruit an additional 25 IBS subjects as well as 25 healthy controls undergoing colonoscopy for clinical purposes (e.g. colon cancer screening) to determine how host-microbial interactions may impact on IBS vs. healthy controls.

Mucosal biopsies will be utilized to generate human intestinal enteroids to study host-microbial interactions determining response to treatment in IBS. Four mucosal biopsies will be obtained from normal appearing mucosa in the ileum, ascending and sigmoid colon (total of 12 mucosal biopsies per patient), placed in a sterile cryovial on ice, and immediately stored at -80°C for long-term storage.

Sequencing and Data Analysis:

(i) MiSeq Illumina Sequencing: DNA samples will be submitted to the University of Michigan Host Microbiome Initiative under the Microbiome Explorers Program and will be processed using the MiSeq Illumina sequencing platform. 16S rRNA gene libraries will be constructed using primers specific to the V4 region.

(ii) OTUs and Diversity Measurements: The open-source software program, Mothur, will be used following the steps outlined in the Mothur MiSeq SOP (66,67). The 16S rRNA gene sequencing profiles will be assessed using metrics for α diversity (such as Shannon or inverse-

Simpson indices) and β diversity-based distance measures between samples, such as θ_{yc} or Bray-Curtis (68,69). Community types will be identified using unsupervised clustering algorithms, and individual operational taxonomic units (OTUs) will be identified and compared between/within patients (see Biostatistical analysis).

(iii) Quantitative PCR Targeting the 16S rRNA Gene: qPCR will be used to quantify the total number of 16S rRNA gene copies/gram. Reactions will be performed on a CFX Connect Real-Time PCR detection system (Bio-Rad Laboratories; Hercules, CA) with universal 16S rRNA gene primers as previously described (33).

Specific Aim 3: Develop a predictive model to identify subsets of IBS-D patients responsive to treatment.

3A. Approach:

We will develop and validate a predictive model of response to low FODMAP diet using data from the subjects randomized to a low FODMAP diet (Aim 1) combined with existing data from a separate cohort of IBS-D subjects previously treated with a low FODMAP diet (HUM 00053274).

3B. Experimental Methods:

We will use the combined cohort of patients treated with a low FODMAP diet (Aim 1 and HUM #00053274), which will be split into a training set from a random sample of 80% of the data while the remaining 20% of the data will serve as the test set. Using a random forest model, 5x2 nested cross-validation (CV) will be performed within the training set for model selection and hyperparameter tuning. The candidate models will be compared by testing for overlap of confidence intervals for area under the curves (AUCs, two-sided test). Once a final model is selected, model performance will be evaluated on the test set.

Study Timeline:

Figure 1 IBS Subject Timeline

Schedule of Events - Low FODMAP Group

Visit Number	Pre-Visit	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5
Visit Description	Pre-screening (Phone/in person)	Baseline (Phone/in person)	Monitoring (Phone/in person)	Monitoring (Phone/in person)	Monitoring (Phone/in person)	Monitoring/ Outcomes (Phone/in person)
Time point	Eligibility	Day 0	Day 7±3 days	Day 14±3 days	Day 28±3 days	Day 35±3 days
7 day pre-screening questionnaire	X					
Informed Consent	X					
Medical History /Physical Examination		X	X	X	X	X
Urine pregnancy test (Females of childbearing age)		X				
Washout	X					
Questionnaires		X		X		X

3-day food diary		X				
24-hour dietary recall			X	X	X	X
GI nutrition consultation		X		X		
Colonoscopy with mucosal biopsies (optional)	X					
Serum Cytokine Profiles (optional)		X				X
Buccal swab collection (optional)		X				
Glucose hydrogen breath testing		X				X
Stool collection for fecal microbial analysis		X (3 baseline samples)		X	X	X
Randomization	X					
Dietary Compliance			X	X	X	X
Adverse Events Monitoring			X	X	X	X

*Female participants capable of bearing children will be required to attend visit 1 in person for the pregnancy test
*During COVID-19 pandemic, all hydrogen breath tests will be conducted remotely

Schedule of Events – Rifaximin Group

*Female participants capable of bearing children will be required to attend visit 1 in person for the pregnancy test
*During COVID-19 pandemic, all hydrogen breath tests will be conducted remotely

Visit Number	Pre-Visit	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5
Visit Description	Pre-screening (Phone/in person)	Baseline (Phone/in person)	Monitoring (Phone/in person)	Monitoring (Phone/in person)	Monitoring (Phone/in person)	Monitoring/ Outcomes (Phone/in person)
Time point	Eligibility	Day 0	Day 7±3 days	Day 14±3 days	Day 28±3 days	Day 35±3 days
7 day pre-screening questionnaire	X					
Informed Consent	X					
Medical History /Physical Examination		X	X	X	X	X
Urine Pregnancy Test (Females of childbearing age)		X				
Washout	X					
Questionnaires		X		X		X
3-day food diary		X				
24-hour diet recall			X	X	X	X
Colonoscopy with mucosal biopsies (optional)	X					
Serum cytokine profiles (optional)		X				X
Buccal swab collection (optional)		X				
Glucose hydrogen breath testing		X				X
Stool collection for fecal microbial analysis		X (3 baseline samples)		X	X	X
Randomization	X					
Medication Dispensing		X				
Medication Compliance			X	X		
Adverse Events Monitoring			X	X	X	X

7 Data Collection and Management Procedures

Data Handling and Entry: All data including questionnaire results and physiologic testing will be recorded and reviewed by the study team. Data will then be entered into an electronic case report form (eCRF) that complies with Title 21 of the Code of Federal Regulations (21 CFR Part 11). All passwords will be strictly confidential to protect subject's protected health information (PHI). Only the Primary Investigator (PI) and Study Coordinator will have access to the data and subject identifiers. Only the PI and Study Coordinator will be allowed to enter data onto the password-protected web-based server.

Computer Systems: Data entry will be entered into REDCAP which is validated and conforms to regulatory requirements. Only the Primary Investigator (PI) and Study Coordinator will have access to the data and subject identifiers. Only the PI and Study Coordinator will be allowed to enter data onto the password-protected web-based server.

Phone Visits: All phone visits will be conducted with a University Health System phone to protect patient confidentiality.

SignNow: a secure application that enables users to electronically prepare and send University business documents for the purpose of requesting and obtaining digital signatures to help protect confidentiality.

Data Validation: Validation checks will be programmed within the eCRF system as well as supplemental validation performed by review of the downloaded data, will be applied to the data in order to ensure accurate, consistent, and reliable data.

Direct Access to Source Data: A developed review procedure that complies with Good Clinical Practice (GCP) guidelines.

Source Document/Case Report Form Completion: Source documents and the eCRFs will be completed for each study patient. It is the Investigator's responsibility to ensure the accuracy, completeness, and timeliness of the data reported in the patient's source document/eCRF. The source document/eCRF should indicate the patient's participation in the study and should document the dates and details of the study procedures, AEs, and patient status.

Record Retention: The Investigator will maintain all study records according to applicable regulatory requirement(s).

8 DATA ANALYSIS

Procedure for Accounting for Missing, Unused, and Spurious Data: The primary outcome measure in our study is changes in mean daily pain or bloating after intervention with rifaximin or low FODMAP diet compared with baseline. We will use multiple imputation with 10 imputed datasets to replace missing values on outcome and predictor variables [77]. If 10 imputed datasets are not sufficient to ensure stability of estimates, we will use 20 imputed datasets. This allows for maximal use of available data while maximizing statistical power.

9 QUALITY CONTROL AND QUALITY ASSURANCE

The PI and Study Coordinator will be responsible for Quality Control and Quality Assurance and will be performed in accordance with Standard Operating Procedures at the University of Michigan.

10 STATISTICAL CONSIDERATIONS

Aim 1:

All of the interventional analyses will be analyzed on an intention-to-treat basis. Each IBS-D subject will act as his/her own control for analysis of the effects of either rifaximin or low FODMAP diet intervention. This paired design inherently eliminates a significant amount of bias/confounding and affords increased statistical power. We will power our study to detect a ≥30% improvement in mean daily bloating or abdominal pain after intervention compared with baseline. Using available data from a US study on low FODMAP diet in IBS patients (42), we conservatively estimate that we will require 14 IBS-D subjects in each arm to have >90% power to detect a mean difference of 1.7 in abdominal bloating scores after intervention via paired t-tests with the expected standard deviation (SD) of ±1.8. Thus, our expected enrollment of 32 IBS-D subjects in each arm will be more than sufficient for analyzing this aim (see biostatistical analysis for Aim 2), even if the SD is nearly two-fold larger than expected.

Aim 2:

We will examine both inter- and intra-individual variability in microbiota-derived metrics. We plan to use a paired design to eliminate a significant amount of bias/confounding and increase statistical power. We anticipate that there will be some variability in the three samples collected prior to treatment, and various techniques will be employed to average these data so they are incorporated as the baseline. For example, for α diversity (Shannon index) a simple average of the three time points will be sufficient to

establish a baseline α diversity. Similar techniques will be applied to other community structure metrics, to create "deltas" between post-treatment time points and the baseline.

These microbe-derived metrics and accompanying clinical variables will be initially analyzed through descriptive statistics before/after intervention. Histograms and measures of frequency or central tendency/spread will be used to optimize variable constructions. Subsequently, initial bivariable longitudinal analyses in an inter-individual fashion (assessing group-wide trends before/after intervention) that also account for the paired nature of the data (intra-individual) will be employed for categorical data (McNemar's test) and continuous data (paired t-test), or their non-parametric equivalents for data not normally distributed. Multiple comparisons will be made for individual OTU abundance metrics, with a focus on the most abundant taxa. However, we will avoid a high false discovery rate through use of linear discriminant analysis effect size (LEfSe) analysis (70). Similar to Aim 1, we will power our study using a paired t-test based on the primary outcome to detect a change in diversity at the end of the intervention compared with baseline. In addition, we will also examine intermediate endpoints or longitudinal trends over time to determine if these are important. If this is the case, we will also use mixed model analyses with repeated measures.

Although this work is exploratory, we wanted to ensure that we will have sufficient power to detect notable differences in microbial and functional characteristics with our planned recruitment base. Based on prior studies with low FODMAP diet in IBS patients (42,45,71), we conservatively estimate that 60% of IBS-D subjects will respond to treatment. We further expect that only those IBS-D subjects who respond to a low FODMAP diet will show changes in fecal microbial diversity. Given our sample size of 32 subjects per arm, we will have >90% power to detect a mean difference ≥ 0.4 in θ_{YC} (a measure of β-diversity) between responders and non-responders. As we are already seeing differences in θ_{YC} of this magnitude or greater, this suggests we are appropriately powered to detect meaningful differences in the microbiota between groups even if our standard deviation is 2-fold greater than expected.

11 REGULATORY REQUIREMENTS

11.1 Informed Consent [1]

[1] The IRB may waive some or all elements of informed consent – consult the IRB for consenting requirements.

Informed consent will be signed by subjects after the protocol is explained by either the PI, co-I or study coordinator. If an IBS-D subject is a patient of the PI or Co-I, the study coordinator will be responsible for obtaining informed consent to avoid potential issues of coercion. Before signing, IBS-D subjects will be given ample opportunity to ask questions about the protocol. Consent forms could be signed in the Taubman Center or in the GI Physiology Laboratory in the Medical Procedures Unit. Please see Informed Consent attached as an appendix.

11.2 Subject Confidentiality

All study team members will make every effort to protect each IBS-D subject's confidentiality. Participants will have privacy in a closed exam room to be able to provide information to the PI and/or study coordinator regarding medical history, current condition, and any other relevant information pertaining to the study. Research records, data, and/or specimens will be kept in a locked office and/or locked cabinet with access restricted only to study team members. Study members will also secure electronic data on computers by using password protection, installing and regularly updating security software, as well as encryption of data. Only the PI and the Study Coordinator will have access to the data and IBS-D subject identifiers. Subjects will receive REDCap encrypted and secure links to fill out the online questionnaires. Only the PI and the Study Coordinator will be allowed to enter data onto the password-protected web-based server. Patients will be called using University Health System phones to protect patient privacy.

11.3 Data Safety Monitoring Plan

11.3.1. Oversight Responsibilities

Oversight of the trial is provided by the Principal Investigator (PI), Dr. Lee. Dr. Lee assures that informed consent is obtained prior to performing any research procedures, that all subjects meet eligibility criteria, and that the study is conducted according to the IRB-approved research plan.

11.3.2 Unanticipated Problems, Adverse Events and Serious Adverse Event Collection and Reporting[2]

[2] The Office for Human Research Protections (OHRP) considers unanticipated problems involving risks to subjects or others to include, in general, any incident, experience, or outcome that meets **all** of the following criteria:

There is minimal direct risk to study subjects as all interventions are well accepted treatments for IBS. All members of the study team will stay current with training in the protection of human research participants and HIPAA. The PI will regularly monitor the data in collaboration with the research team, with periodic review by the IRB at the University of Michigan. We will minimize risk by using rigorous data security protocols to protect protocol deviations (e.g. violation of human subject confidentiality or comprised data integrity) and will ensure expedient reporting to the IRB (see below).

All adverse clinical experiences, whether observed by the investigator or reported by the subject, must be recorded, with details about the duration and intensity of each episode, any actions taken, and the subject's outcome. The Principal Investigator must evaluate each adverse experience for its relationship to the study and for its seriousness.

The Principal Investigator must appraise all abnormal laboratory results for their clinical significance. If any abnormal laboratory result is considered clinically significant, the Principal Investigator must provide details about the action taken and about the subject's outcome.

All AEs will be assessed by the Investigator(s) and will be recorded in the eCRF, including the date of onset and resolution, severity, relationship to study, outcome, and action taken. All AEs will be reported to the IRB that meet reporting criteria. Toxicity will be graded according to the Common Toxicity Criteria as follows:

0 = No adverse event or within normal limits

1 = Mild AE, not requiring treatment

2 = Moderate AE, resolved with treatment

3 = Severe AE, resulted in inability to carry on normal activities and required professional medical attention

4 = Life threatening or disabling AE

- unexpected (in terms of nature, severity, or frequency) given (a) the research procedures that are described in the protocol-related documents, such as the IRB-approved research protocol and informed consent document; and (b) the characteristics of the subject population being studied;
- related or possibly related to participation in the research (in the guidance document, possibly related means there is a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures involved in the research); and
- suggests that the research places subjects or others at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or recognized.

An incident, experience, or outcome that meets the three criteria above generally will warrant consideration of substantive changes in order to protect the safety, welfare, or rights of subjects or others.

5 = Fatal AE

Serious adverse event (SAE) is defined by the FDA as an AE resulting in any of the following outcomes: Death, a life-threatening adverse event, inpatient hospitalization or prolongation of existing hospitalization, a persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions, or a congenital anomaly/birth defect. Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered serious when, based upon appropriate medical judgment, they may jeopardize the patient or subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition. Once an AE or SAE is known, research team members at the study site will ensure that participants receive appropriate care and all actions taken by the PI and/or co-investigator after observing the AE or SAE will be documented.

11.3.2. Management of Risks to Subjects

Expected AEs:

Expected AEs associated with the interventions being used in the study and study procedures include:

- Abdominal cramping, bloating, flatulence, lightheadedness or dizziness with the GHBT
- Pain, bruising or lightheadedness with the blood draw

AE Management:

Subjects will be allowed to take medications, such as simethicone or Tylenol, if they experience significant bloating or discomfort with the GHBT. They will also be offered juice or other beverages if they experience dizziness or lightheadedness with the GHBT or with blood draws.

11.3.3 Entity Responsible for Monitoring

The Principal Investigator is responsible for complying with local and institutional requirements related to the reporting and documenting of SAEs. These reports must include submission of all qualifying SAEs to the IRB and/or Independent Ethics Committee (or others). The guidelines established in the Committees on Human Research “Adverse Event and Unanticipated Problems Reporting Policy” will be followed.

11.3.3 Data Analysis Plans

Study data are accessible at all times for the PI to review. The PI reviews study conduct, including accrual, drop-outs, and protocol deviations, on a monthly basis. The PI reviews AEs individually real-time and in aggregate on a quarterly basis. The PI reviews serious adverse events (SAEs) in real-time. The PI ensures all protocol deviations, AEs, and SAEs are reported to the IRB according to the applicable regulatory requirements.

11.3.4 Stopping Rules

This study will be stopped prior to its completion if: (1) the intervention is associated with adverse effects that call into question the safety of the intervention; (2) difficulty in study recruitment or retention will significantly impact the ability to evaluate the study endpoints; (3) any new information becomes available during the trial that necessitates stopping the trial; or (4) other situations occur that might warrant stopping the trial.

11.3.5 Safety Review Plan

Study progress and safety will be reviewed monthly (and more frequently if needed). An Annual Report will be compiled and will include a list and summary of AEs. In addition, the Annual Report will address (1) whether AE rates are consistent with pre-study assumptions; (2) reason for dropouts from the study; (3) whether all participants met entry criteria; (4) whether continuation of the study is justified on the basis that additional data are needed to accomplish the stated aims of the study; and (5) conditions whereby the study might be terminated prematurely. The Annual Report will be forwarded to the IRB. The IRB and other applicable recipients will review progress of this study on an annual basis.

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Appendix 1

Cytokine Profile Collection:

Visits 2 and 6 (if optional consent is obtained):

1. 10 ml of blood will be drawn and placed into 2 SST tubes.
2. Whole blood SST tubes will be transported at room temperature to the lab.
3. Processing for SST tubes:
    a. SST tubes will be allowed to clot at room temperature for 30 minutes.
    b. Serum will be separated from the red blood cells by spinning for **15 min at 1300G at 4°C**.
    c. The serum will then be aliquoted from each blood collection tube into (3) 2-ml transfer tubes and then the collection tube will be discarded into the red biohazard disposal bucket.
    d. Each collection tube will be labeled with the subject's unique ID number and/or letters.
    e. Each collection tube will then be frozen at -80°C for future analyses.